

Characterization of neuropeptide F and its receptor from the African malaria mosquito, *Anopheles gambiae*

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Abstract

The genome of *Anopheles gambiae* contains sequences encoding a neuropeptide F (Ang-NPF) and NPF receptor (Ang-NPFR) related to the neuropeptide Y signaling family. cDNAs for each were cloned and sequenced. Ang-NPFR was stably expressed for radioligand binding analysis. Ang-NPF exhibited high affinity ($IC_{50} \sim 3$ nM) membrane binding; NPFs from *Aedes aegypti* (Aea-NPF) and *Drosophila melanogaster* (Drm-NPF) were less potent, with the rank order: Ang-NPF > Aea-NPF > Drm-NPF > Drm-NPF_{8–36}. RT-PCR analysis revealed Ang-NPF and Ang-NPFR transcripts in all life stages. Ang-NPF and Ang-NPFR may be strategically positioned for signaling in relation to nutritional status in the African malaria mosquito.

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1. Introduction

Members of the neuropeptide Y (NPY) family of regulatory peptides have a broad occurrence among metazoan animals. Among vertebrates, family members include NPY, peptide YY (PYY), and pancreatic polypeptide (PP) (cf. [2]). Orthologous peptides have been characterized for a wide range of invertebrate taxa, including platyhelminths, mollusks, and arthropods [30]. These invertebrate peptides have been termed neuropeptide Fs (NPF). Sequence similarities among NPY family members are readily apparent across the taxonomic spectrum (cf. [30]), with the closest similarities coinciding with species relatedness. Among insects, NPFs have been identified by sequence for the fruit fly, *Drosophila melanogaster* (Drm-NPF; [3]), the yellow fever mosquito, *Aedes aegypti* (Aea-NPF; [30]), and the African malaria mosquito, *Anopheles gambiae* (Ang-NPF; [24]).

Receptors for NPY family members have been characterized for many vertebrates, but only a few invertebrates. All are seven-transmembrane G-protein coupled receptors (GPCR). For mammals studied to date, five subtypes of receptors appear to occur in each species [2]. Among invertebrates, receptors for NPFs have been identified functionally only for a snail, *Lymnaea stagnalis* [34] and for *D. melanogaster* [12]. Although sequence similarities among NPY receptors across taxa are apparent [8,11–13], the small number of sequences available for invertebrates has limited phylogenetic analyses. Genome sequencing of select invertebrates provides rapid advances [13,18,20,33,35,36], and the recent completion of the genome of *A. gambiae* augmented knowledge of GPCRs [14], including those resembling identified NPY receptors.

Knowledge of the physiological actions of peptides in the NPY family is expanding. Among mammals, NPY, PYY, and PP exert regulatory roles in food intake, digestion, metabolism, and reproduction [2], among other activities. Particularly intriguing are the indications that NPY elicits the orexigenic response of increased food intake centrally, whereas PYY secreted from the gut inhibits food intake [22].

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The latter anorexigenic effect is mediated by PYY_{3–36}, the principle circulating form of PYY, which acts via the NPY Y₂ receptor subtype [1]. Sequences of invertebrate NPF receptors most closely resemble those of the Y₂ subtype (see below). For insects, the localization of NPF in the central nervous system (CNS) and in midgut [3,30] resembles the distribution of NPY family peptides in vertebrates.

For dipteran insects, a role for NPF in coordination of physiological processes dependent on nutritional status is suggested by initial studies in *D. melanogaster* [28,39] and *A. aegypti* [30]. In female *A. aegypti*, the hemolymph titer of immunoreactive NPF is altered by consumption of a blood meal [30]. A blood meal is required for initiation of vitellogenesis, completion of oogenesis, and consequent reproductive success. As for many insect vectors, diseases are spread through feeding activities. For *A. gambiae*, hematophagy underlies the transmission of *Plasmodium* parasites and the perpetuation of malaria.

In the present study, Ang-NPF and a candidate NPF receptor (Ang-NPFR) were identified in the database of the *A. gambiae* genome, and cDNAs for each were cloned and sequenced. The cloned Ang-NPFR was transfected for stable expression in CHO-K1 cells for radioligand binding analysis. Species specificity of receptor binding was studied by comparing binding activities of Ang-NPF, Aea-NPF, and Drm-NPF. The distribution of Ang-NPF and Ang-NPFR transcripts in differing stages and body regions was determined by RT-PCR. Results indicate that Ang-NPFR is likely the cognate receptor for Ang-NPF and that the ligand and receptor may be strategically positioned for signaling in relation to nutritional status in the African malaria mosquito.

2. Experimental procedures

2.1. Cloning and sequences analysis of Ang-NPFR cDNAs

To obtain cDNA, total RNA was extracted from ~250 frozen larvae using the RNeasy[®] mini RNA extraction kit according to the manufacturer's instructions and included DNase treatment (Qiagen). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a two-step protocol employing the following condition: mRNA in total RNA from larvae was converted to cDNA with pd(T)_{12–18} oligo dT primer for 30 min at 42 °C by reverse transcription using a first strand synthesis kit (Amersham), then inactivated at 95 °C for 5 min.

Nucleotide primers specific for genes encoding Ang-NPF and the putative Ang-NPFR were designed to encompass the deduced open reading frames (ORF). The primer pairs had the following sequences: Ang-NPFFwd—GAC GAT GGC GTC AGG CAC and Ang-NPFRrev—CAT GTC TAG ATA TTT GTT GGT AGC TG; Ang-NPFRfwd—GCA CAA CCG TCA TCC ACC ATG G and Ang-NPFRrev—CGG GAC ATC AGG ACA TCA GCT C. Larval cDNA was used as tem-

plate (1 μl) in PCR for the amplification of products encoding the Ang-NPF ORF with the appropriate primers (0.2 pmol each primer; 0.2 μM each dNTP) and Taq Titanium polymerase (BD; 1 U/50 μl total volume with 1X buffer) using an Eppendorf Mastercycler gradient thermal cycler: initial denaturation for 5 min at 95 °C, then amplification for 30 s at 95 °C, 30 s at 72 °C/cycle, 30 s at 72–1 °C for 14 cycles, then 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C for 25 cycles followed by a 10 min 72 °C incubation. Ang-NPFR ORF products were amplified from larval cDNA with the appropriate primer pairs using the Expand High Fidelity PCR system (Roche, 2.6 U/50 μl; PCR mixture as above) according to the manufacturer's instructions: initial denaturation for 5 min at 95 °C, then amplification for 15 s at 95 °C, 30 s at 56 °C, 75 s at 72 °C for 10 cycles, then 15 s at 95 °C, 30 s at 56 °C, 75 + 5 s/cycle at 72 °C for 25 cycles followed by a 10 min 72 °C incubation.

PCR products were separated on 1% agarose gels, and ones of the expected size were excised, purified using GenElute minus EtBr spin columns (Sigma), and cloned into pCR[®]II-TOPO with TOP 10 *Escherichia coli* competent cells (TOPO TA cloning[®] kit; Invitrogen, Carlsbad, CA). After cloning, 20–50 white colonies were picked, and plasmid DNA purified (QIAprep[®] spin miniprep kit, QIAGEN Inc., Valencia, CA). Four or more different Ang-NPF and Ang-NPFR clones were sequenced at the Molecular Genetics Instrumentation Facility (MGIF, University of Georgia, Athens, GA) to obtain a single consensus nucleotide sequence. After it was translated into a suitable amino acid sequence, prediction of signal sequence, transmembrane domains, O-glycosylation sites, and Ser/Thr/Tyr phosphorylation sites were done using the CBS prediction servers (Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, www.cbs.dtu.dk). Ang-NPFR, Drm-NPFR, and NPY receptor family sequences obtained from GenBank were subjected to Clustal X alignment, and a tree was constructed based on Jukes–Cantor distance analysis (GCG), for an initial perspective on receptor phylogeny.

2.2. RT-PCR analysis of Ang-NPF and Ang-NPFR gene expression in eggs, larvae, pupae and adults

Total RNA was extracted separately from 300 eggs and from dissected head, thorax or abdomen of 10 individuals, each of 4th instar larvae, pupae, adult males, and non-blood fed adult females, using the RNeasy[®] mini RNA extraction kit as described above. mRNA in the total RNA samples was converted to cDNA primed with pd(T)_{12–18} oligo dT primer using Advantage reverse transcriptase for PCR (BD Biosciences) according to the manufacturer's instructions. As described above for Ang-NPF RT-PCR, products were amplified by PCR from each of the cDNAs obtained from the different life stages and body regions with the Ang-NPF primer pair, Ang-NPFFwd and Ang-NPFRrev, or the Ang-NPFR pair, Ang-NPFRfwd and Ang-NPFRrev (ATCGC AGT GAT GGA TAT GGT TGA CAC). PCR products were separated on 1.2%

agarose gels, and bands were photographed. Initially, identification of the PCR products was confirmed by sequencing.

2.3. Expression of putative Ang-NPF receptor cDNAs in CHO-K1 cells

The ORF coding region of Ang-NPFR cDNA was excised from pCR[®]II-TOPO using the restriction endonuclease *EcoRI*. The purified insert was cloned into the mammalian expression vector pcDNA3.1+ (Invitrogen, Carlsbad, CA) linearized with the same enzyme, and transformants were analyzed by PCR for directionality. The cDNA construct was used to transfect CHO-K1 cells (American Type Culture Collection, CCL-61) according to the protocol for six well tissue culture plates using LIPOFECTAMINE reagent (Life Technologies Inc.). Briefly, CHO-K1 cells were grown in RPMI-1640 media (Fisher) containing 1 mM pyruvate (Fisher) and 10% fetal bovine serum (FBS; Fisher Scientific) at 37 °C with 6% CO₂ in six well tissue culture plates to a density of approximately 75% confluency. DNA complexes were formed by adding LIPOFECTAMINE reagent to the cDNA constructs (1 µg) diluted in RPMI-1640 media. The DNA-LIPOFECTAMINE reagent complexes were added to the CHO-K1 cells containing RPMI-1640 media and incubated for 3 h at 37 °C with 6% CO₂. After 3 h, RPMI-1640 media containing 1 mM pyruvate and 10% FBS was added, and the cells were incubated for 48 h at 37 °C with 6% CO₂. For stable expression of Ang-NPFR, the transfected CHO-K1 cells were selected for 3 weeks with 800 µg/ml G418 (Fisher) added to the tissue culture media. Expression of Ang-NPFR cDNA in CHO-K1 cells was determined by RT-PCR as described above. CHO-K1 cells expressing Drm-NPFR (previously DmNPFR1, CG1147) were prepared as previously described [12].

2.4. Peptides and iodinations

The amino acid sequences for Ang-NPF, Aea-NPF, and Drm-NPF were deduced from their cloned and sequenced cDNAs [3,24,30], and chemically synthesized (Ang-NPF, LVAARPQDSDAASVAAAIRYLQELETKHAQHARPRFamide, Dr. Kevin Clark, University of Georgia, Athens, GA—expected 3985.1 Da confirmed by mass spectroscopy; Aea-NPF: SFTDARPQDDPTSVAEAIKLLQELETKHAQHARPRFamide, Dr. Stephan Klauser, University of Zurich Hospital, Zurich, Switzerland; Drm-NPF: SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFamide, >85% pure; Drm-NPF₈₋₃₆: KNDVNTMADAYKFLQDLDTYYGDRARVRFamide, >85% pure; Quality Controlled Biochemicals Inc., Hopkinton, MA). According to the method established for bovine PP ([38]; see also [6]), Ang-NPF and Drm-NPF each were iodinated by mixing 10 µg synthetic peptide, 500 µCi Na¹²⁵I (Amersham), 400 ng lactoperoxidase and 10 µl 0.006% hydrogen peroxide in 95 µl 200 mM sodium phosphate buffer, pH 7.5. After incubation at RT for 10 min with occasional shaking, an

additional 10 µl of 0.006% hydrogen peroxide was added, and after 10 min the reaction terminated by adding 100 µl of 50% acetic acid. The radiolabeled Ang-NPF or Drm-NPF mixture was loaded into a Beckman HPLC 421A/110B system and fractionated on a reverse phase C₈ column (Vydac, 300 Å, 4.6 mm × 150 mm): solvent A, water with 0.1% trifluoroacetic acid; and solvent B, 80% CH₃CN in solvent A with gradient program: 0–57% B, 10 min; 57–60% B, 50 min; 60–100% B, 10 min at 1 ml/min. ¹²⁵I-labeled Ang-NPF and Drm-NPF were detected with an in-line Beckman Model 170 radioisotope detector, fractions containing the radiolabeled peptide were collected, and bovine serum albumin (BSA; Fraction V; Sigma) was added to 1%. The fractions containing the radiolabeled peptide were counted on a Beckman Gamma 4000; final concentrations were determined based on specific activity (~2000 Ci/mmol) of ¹²⁵I in the product, which was free of unlabeled peptide and assumed to be monoiodinated.

2.5. Membrane preparation and peptide binding assays

Membranes were prepared from CHO-K1 cells transfected with pcDNA3.1+ (control), Ang-NPFR or Drm-NPFR by differential centrifugation. CHO-K1 cell lines expressing Ang-NPFR or Drm-NPFR were seeded into 75 cm² tissue culture flasks and grown to confluence in RPMI-1640 media containing 10% FBS and 1 mM pyruvate at 37 °C in 6% CO₂. After reaching confluency, cells were washed two times with phosphate buffered saline (PBS; Fisher Scientific), and then cells were scraped from the flasks in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose with 1 protease inhibitor tablet/10 ml; Roche). The scraped cells, in homogenization buffer, were transferred to Oak Ridge tubes (on ice), then homogenized, and centrifuged at 2000 × g for 10 min at 4 °C. The supernatant was transferred to a fresh Oak Ridge tube and then centrifuged at 48,000 × g for 1 h at 4 °C to pellet the membranes. The resulting membrane pellet was resuspended in homogenization buffer, and the membranes sheared through a 25-ga needle, and then frozen at –80 °C until used in binding assays.

To assess NPF binding, membranes were added to binding buffer (50 mM Tris-HCl, pH 7.5, 1X Hanks' Balanced Salt Solution, 1% BSA, with 1 protease inhibitor tablet/10 ml), containing 100 pM ¹²⁵I-Ang-NPF or ¹²⁵I-DmNPF along with various concentrations of Ang-NPF, Aea-NPF, or Drm-NPF as competitor, and then incubated for 3 h at RT. After the incubation, membranes were recovered by centrifugation (14,000 rpm at 4 °C), and then washed two times with ice-cold PBS to remove unbound ¹²⁵I-Ang-NPF or ¹²⁵I-DmNPF. Tubes were counted on a Packard CobraII gamma counter. The raw counts obtained from the binding assays were converted to percent binding, and these data analyzed by non-linear regression analysis (v3.0 GraphPad Software Inc., San Diego, CA) to obtain curves, IC₅₀ values (concentration of Ang-NPF, Aea-NPF or Drm-NPF that

(A)

1 **GACGATGGCGTCAGGC**ACTTTTACTCAGCGTTTGCTGGTGGCTCTTATGATATTCGCCCTGATGCCGACCTTAGCACACTAGTTGCAGCCCGACCCGAAGA
 M A S G T F T Q R L L V A L M I F A L I A D L S T L V A A R P Q D
 10 20 30

103 TAGTGACGCTGCTCTGTAGCTGCCGAATTAGATACCTCCAGGAGCTTGAACCTAAGCATGCCAACATGCTAGACCCAGATTCCGAAAGCGTGGTGGATA
 S D A A S V A A A I R Y L Q I L E T K E A Q H A R P R F G **K R** G G Y
 40 50 60

205 TCTTAACCCGGCAATATTTGGGCAGGATGAACAGGAGAATCTTTACAGGTTGATTGGCAGGATTCAACATTTTCGAGATGAAC**CAGCTACCAACAAATATCTA**
 L N P A I F G Q D E Q E N L Y R L I G R I Q H F R D E Q L P T N I *
 70 80 90 100

307 **GACATG**

(B)

1 **GCACAACCGTTCATCCACCATGGAATCCG**TACTGACCCGCTTCAATCTCACGCTCGACAATATGACAACGTTAAGCTCCAACATACGCCAGGGGCTTATCGAG
 M E S V L T R F **N L T** L D **N M T** T L S S N I R Q G L I E
 10 20

103 CAGTACAGCAACAATCGGAAGGTGGCCGATCCCTGGTACCATATCTTGATCATCATGTACGGTACGCTGATCGTGTTCGGCGCTACAGGGAACAGTCTGGTG
 Q Y S N N R K V A D P **Y H** I L I I M Y G T L I V F G A T G N S L V
 30 40 50 TM1 60

205 GTGTGGCCGTGCGCCGAAGCCACAGATGCGAACCGCTCGCAACATGTTTCATCGTGAATTTGGCCGTTTCCGATCTACTACTGTGCTAGTGACGATGCCA
 V L A V A R K P Q M R T A R N **M F I V N L A V S D L L L C L V T M P**
 70 80 TM2 90

307 CTGACGCTGGTGGAAATCTCACCAAATACTGGCCGATGGGTCGGTTACCATTTTTGTGCAAAATCGATCGGCACACTACAAGCTACCAGCATATTC**GTGTCA**
 L T L V E I L T K Y W P M G R L P F L C K S I G T L Q A T S I F V S
 100 180 120 TM3 130

409 **ACCATATCCATCACTGGGATCGCA**TTAGATAGGTATCAAGTTAAGTTATCCGACTCGGACAGTCTACAGCTGATGGGTGCAATCGCTATCTTGACCGGT
 T I S I T A I A L D R Y Q V I V Y P T R D S L Q L M **G A I A I L T G**
 140 150 160

511 ATCTGGATCATCTCCATAGTACTAGCTTCGCCGATGTTTATCATCCGGAACCTAATCCACTACGACGCTCAATCTGCCAGCCTCGGGATCGAGTACGTGTGG
 I W I I S I V L A S P M F **I** R Q L I H Y D V N L P S L G I E Y V S
 TM4 100 110 190

613 TACTGCATCGAGGACTGGCCGATTGCATACGGCCGTGTGTACTATTCCGGCTTCACTCTTTGCGTGCAGTACGCTGCTACCCATTCTGATCGTGTGATGGCA
 Y C I E D W P I A Y G R V **Y** Y S A F T L C V Q Y V L P I L **V S M A**
 200 210 260 TM5 290

715 TACCTGCGCATCTATCTGAAGCTAAAGCACCGGCTAGTGGTGGGACGGCCAGCGGAAGCCCGGGAAGCGAAACCCGTGCGGGAGCGGGAACGTGGCCGG
 Y L R I Y L **L** **H R L V V G T A S G K P G E A K P V R E R E R G R**
 240 250 260

817 CGGATGCAACGCACCAACTATCTGTGATCAGCATTGCGCTAATCTTTGGCGTTTCTGGCTACCACTCAATCTGTTCAATCTGTTCCGGACCTGTACGTG
 R M Q R T N **Y L L I S I A L I F G V S W L P L N L F N L F A D L Y V**
 270 280 TM6 290 300

919 CACTCGATCAGCAGGACATTATGGTGGCGTACGCAATCTGTACATGGCTGGCATGAGCTCGGCCTGCTCCAATCCACTGCTGTACGGCTGGTTGAATGAC
 H S I T Q D **I M V A Y A I C H M A G M S S A C S N P L L Y G W L N D**
 310 TM7 320 330

1021 AATTTTCGGAAAGAGTTCAACGAACCTGCTTCCGGACGCTCGGCGGGCGCCGGGCACGGATCGGGCGGTCACTCGAACGGAAGCCGAGCGAATGGAGGA
 N F R K E F N E L L C R T S A G G P G H G S G G H S N G S R A N G G
 340 350 360

1123 GCTGCCACGGTTGGACGAACGCGAGCTGCTCGAAGTGGACCCGACGGTGGACCTGACGACCCGACGCTGCCGGAACCTAACGCAGGTGCGCGACCGTATGCG
 A A T V G R T R A A R T A **D G G P D D R T L P E L T Q V R D R H A**
 370 380 390 400

1225 GCAGCACTGCTGCAGCACTCGGGCATTACCGAGAACGGTGACCATACTGAGCTGAC**CAGCTGATGCTCTGATGCTCCG**
 A A L L H D S G I T E N G D H T E L T E L M S *
 410 420

Fig. 1. Nucleotide and deduced amino acid sequences for the open reading frames (ORF) of cDNAs encoding *A. gambiae* NPF (Ang-NPF; GenBank AY579077) and NPF receptor (Ang-NPFR; GenBank AY579078). (A) Ang-NPF ORF with the amino acid sequence for the prepropeptide: putative signal peptide in bold italic, processed form of NPF underlined, and convertase cleavage site boxed to indicate C-terminal cleavage and subsequent enzymatic conversion of Gly₆₂ to a C-terminal amide. Oligonucleotide primers for RT-PCR in bold letters. (B) Ang-NPFR ORF with the predicted seven transmembrane domains underlined in the protein sequence. Potential *N*-glycosylation sites boxed and potential intracellular Ser/Thr phosphorylation sites in bold letters. Oligonucleotide primers for RT-PCR in bold letters.

reduces specific binding of labeled ligand by 50%) and statistics, including values of R^2 and standard errors.

3. Results

3.1. Nucleotide sequence and expression of *A. gambiae* NPF and NPF receptor

Putative DNA sequences encoding NPF and its receptor were identified in the *A. gambiae* genome [14,24]. Larval cDNAs and nucleotide primers specific for the predicted ORFs of Ang-NPF and Ang-NPFR were used to amplify by PCR products that were TA-cloned and sequenced (Fig. 1). For Ang-NPF (GenBank AY579077), the prepropeptide encoded by the ORF has 100 amino acids of which the first 25 amino acids constitute a putative signal peptide, based on findings for Aea-NPF [30]. After removal of the signal peptide, proteolysis at a dibasic residue site, Lys₆₃ and Arg₆₄ would yield a peptide of 37 amino acids, and enzymatic amidation of the C-terminal Gly₆₂ would result in a mature peptide form with 36 amino acids (Fig. 1A); the remaining C-terminal of the prepropeptide is similar to that observed for Drm-NPF [3]. The deduced protein encoded by the ORF of Ang-NPFR (GenBank AY579078) is 425 amino acids (Fig. 1B). This protein is predicted to have seven transmembrane domains along with the corresponding intracellular and extracellular loops, consistent with known GPCRs. The N-terminal extracellular region of Ang-NPFR exhibits two potential *N*-glycosylation sites, along with four potential intracellular Ser/Thr phosphorylation sites: one Ser on the third intracellular loop and one Ser and two Thr on the C-terminal intracellular tail (cf. Fig. 1).

Transcript expression patterns of Ang-NPF and Ang-NPFR were determined for eggs, and head, thorax and abdomen regions of fourth instar larvae, pupae, adult males and females by RT-PCR. The 310 bp PCR product for the Ang-NPF ORF was detected in eggs, and head, thorax, and abdomen of fourth instar larvae and pupae; and, head and

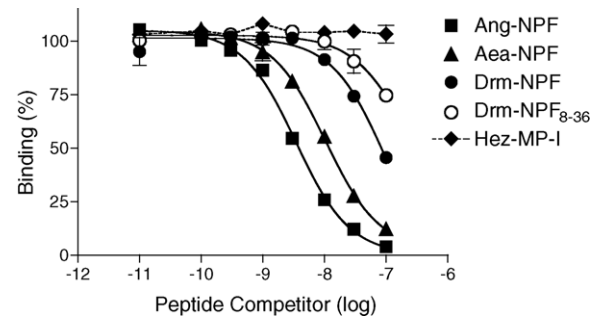


Fig. 3. Competitive inhibition of ^{125}I -Ang-NPF binding to membranes prepared from CHO-K1 cells stably transfected with Ang-NPFR cDNA. Membranes were incubated with 100 pM ^{125}I -Ang-NPF and various concentrations of Ang-NPF, Aea-NPF, Drm-NPF, Drm-NPF₈₋₃₆, or Hez-MP-I for 3 h at room temperature. Values indicate means \pm S.E. ($N=3$).

abdomen of adult males and females (Fig. 2 upper panel). A minor PCR product of 431 bp was also detected in these samples, and its sequence was that of an incomplete splice product of the Ang-NPF gene with an intron of 121 bp (data not shown). This intron is located between nucleotides 183 and 184 of the mature cDNA (cf. Fig. 1A), and contains the consensus donor 'GT' and acceptor 'AG' splice motif (data not shown). RT-PCR for Ang-NPFR showed a pattern of PCR products essentially the same as above, except that the PCR product was not present in eggs, and only a faint band was detected in abdomens of adult males (Fig. 2 lower panel).

3.2. Analysis of NPF Binding to Ang-NPFR and Drm-NPFR

To determine if Ang-NPFR is a functional receptor for Ang-NPF, a radioreceptor approach was taken. Membranes prepared from CHO-K1 cells expressing the potential *A. gambiae* NPFR cDNA specifically bound ^{125}I -Ang-NPF, and this binding was displaced by the addition of Ang-NPF in a concentration-dependent manner (Fig. 3). An IC_{50} of 3.48 nM was calculated from the binding data. In addition, Aea-NPF, Drm-NPF, Drm-NPF₈₋₃₆, and *Heliothis zea*

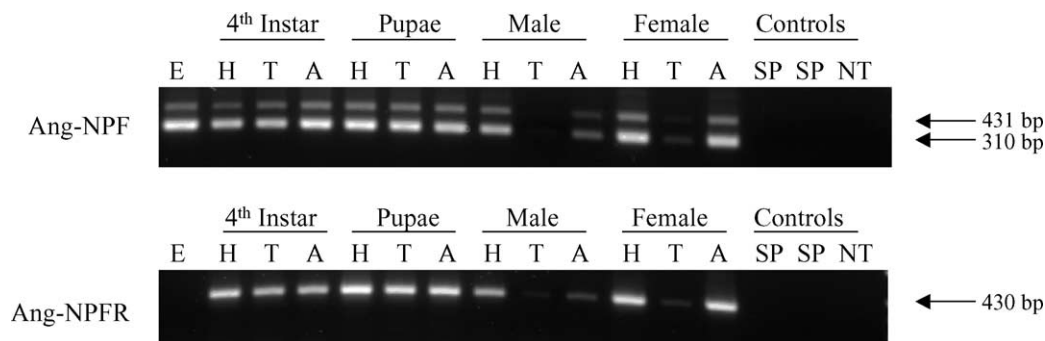


Fig. 2. RT-PCR detection of PCR products indicating transcription of the *Ang-NPF* and *Ang-NPFR* genes in life stages of *A. gambiae*. Nucleotide primers spanning the ORF of Ang-NPF (upper panel) and primers spanning an intron of Ang-NPFR (lower panel) were used to amplify products (310 bp expected for Ang-NPF; 430 bp for Ang-NPFR) from cDNA templates obtained from 36 h eggs (E) and the head (H), thorax (T), and abdomen (A) of 4th instar larvae, pupae, 3-day-old males and 3-day-old sugar-fed females. The 431 bp product seen in the Ang-NPF RT-PCR is an incomplete splice product of the *Ang-NPF* gene with an intron of 121 bp. For controls, SP indicates single primer and NT indicates no template.

Table 1

IC₅₀ values of dipteran NPFs on *A. gambiae* NPFR and *D. melanogaster* NPFR

	Ang-NPFR	Drm-NPFR
Ang-NPF	3.48 (2.98–4.07; $R^2 = 0.99$) ^a	8.13 (6.7–9.87; $R^2 = 0.99$)
Aea-NPF	10.4 (8.74–12.4; $R^2 = 0.99$)	87 (39.7–190; $R^2 = 0.95$)
Drm-NPF	96.9 (42.4–222; $R^2 = 0.95$)	1.64 (1.22–2.21; $R^2 = 0.98$)
Drm-NPF _{8–36}	202 (12.1–3362; $R^2 = 0.80$)	10.6 (7.69–14.6; $R^2 = 0.98$)
Hez-MP-I	NA	NA

^a IC₅₀ values indicate mean (95% confidence interval; R^2 value; $N = 6$); NA, not active.

Amino acid sequences of dipteran members of the NPF family.

	* * * * * * * * * * * * *
Ang-NPF	LVAARPDSDAASVAAATRLQLELETKHAQHARPRFa
Aea-NPF	SFTDARPDPTPSVAEATRLQLELETKHAQHARPRFa
Drm-NPF	SNSRPERKNDVNTIMADAYKELQDLDTYYCDRARVREa

■ indicates majority identical; ▨, majority conserved; *, all identical; *, all conserved

Fig. 4. Amino acid sequence comparisons for NPFs from *A. gambiae*, *A. aegypti*, and *D. melanogaster*.

midgut peptide (Hez-MP-I; QAARPRFa) were used as competitors of ¹²⁵I-Ang-NPF in binding assays to determine if they would bind to the Ang-NPFR. Each of the dipteran NPFs displaced ¹²⁵I-Ang-NPF in a concentration-dependent manner, while no displacement was observed for Hez-MP-I (Fig. 3). The rank order of potency for the peptides was Ang-NPF > Aea-NPF > Drm-NPF > Drm-NPF_{8–36} (Table 1), consistent with their sequence similarities (Fig. 4). Membranes prepared from CHO-K1 cells stably transfected with pcDNA3.1+ vector alone exhibited no specific binding for ¹²⁵I-Ang-NPF (data not shown).

Reciprocal binding assays were done to determine if the mosquito NPFs interact with the *D. melanogaster* NPFR. As expected from previous binding assays employing whole cells [12], membranes from CHO-K1 cells stably transfected with Drm-NPFR specifically bound ¹²⁵I-Drm-NPF, and this binding was displaced by the addition of Drm-NPF in a concentration-dependent manner (Fig. 5). An IC₅₀ of 1.64 nM was calculated from the binding data. Ang-NPF, Aea-NPF, Drm-NPF_{8–36}, and Hez-MP-I were used as competitors of ¹²⁵I-Drm-NPF in binding assays to determine whether they would bind to the Drm-NPFR. Each of the dipteran NPFs displaced ¹²⁵I-Drm-NPF in a concentration-dependent manner, while no displacement was observed for Hez-MP-I (Fig. 5). The rank order of potency for the peptides was Drm-NPF > Ang-NPF ~ Drm-NPF_{8–36} > Aea-NPF (Table 1).

4. Discussion

The present study indicates that the African malaria mosquito, *A. gambiae*, possesses a neuropeptide F, Ang-NPF,

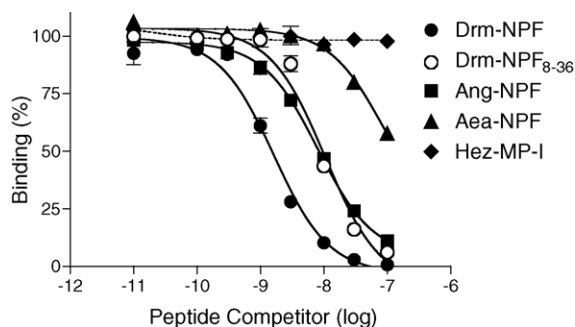


Fig. 5. Competitive inhibition of ¹²⁵I-Drm-NPF binding to membranes prepared from CHO-K1 cells stably transfected with Drm-NPFR cDNA. Membranes were incubated with 100 pM ¹²⁵I-Drm-NPF and various concentrations of Ang-NPF, Aea-NPF, Drm-NPF, Drm-NPF_{8–36}, or Hez-MP-I for 3 h at room temperature. Values indicate means ± S.E. ($N = 3$).

and its receptor, Ang-NPFR, which are members of the larger family of NPY peptides and receptors. The earlier identification of Ang-NPF *in silico* [24] now has been confirmed directly by cloning and sequencing its cDNA. The sequence of Ang-NPF resembles that of Aea-NPF and Drm-NPF (Fig. 4), as well as other NPY family members (cf. [30]). Compared to vertebrates, the Ang-NPFR is structurally most similar to the mammalian NPY family receptors of the Y₂ subtype (Fig. 6), which recently have been shown to mediate the anorexigenic effects of PYY_{3–36} released from the gut in response to eating [1]. Whether Ang-NPF acts on Ang-NPFR to exert a similar effect in this mosquito is not yet known.

The genome of *A. gambiae* contains three seven-transmembrane GPCRs which are related by sequence to the NPY receptor family (Fig. 6). One of these, Ang-NPFR, appears to be a bona fide receptor for Ang-NPF. This peptide displaces radiolabeled Ang-NPF with apparent high affinity (IC₅₀ ~ 3 nM). The sequence of Ang-NPFR most closely resembles Drm-NPFR and the *Lymanaea* NPYR (see also [11]). The latter two receptors previously have been shown to be functional receptors for the NPF of *D. melanogaster* [12] and of *L. stagnalis* [34], respectively. Moreover, the NPFs of *D. melanogaster* and *L. stagnalis* also were shown to inhibit adenylyl cyclase activity when the respective peptides were added to CHO-K1 cells expressing these receptors [12,34]; such inhibition is prototypical for members of the larger NPY receptor family. Accordingly, Ang-NPFR likely exhibits a similar signaling characteristic.

Sequences now have been determined for NPFs from three species of dipterans, *A. gambiae* ([24]; and the present study), *A. aegypti* [30], and *D. melanogaster* [3]. For the two species of mosquitoes, amino acid sequence identity is notable in the carboxyl terminal two-thirds of their NPFs (Fig. 4). A similar pattern typifies comparisons to Drm-NPF, although sequence relatedness is reduced compared to either mosquito NPF. Intriguingly, the Drm-NPF first isolated from *D. melanogaster* for sequencing was Drm-NPF_{8–36} [3]. Given the existence of cell lines each stably expressing Ang-NPFR and Drm-NPFR, it was of interest to assess the species specificity of

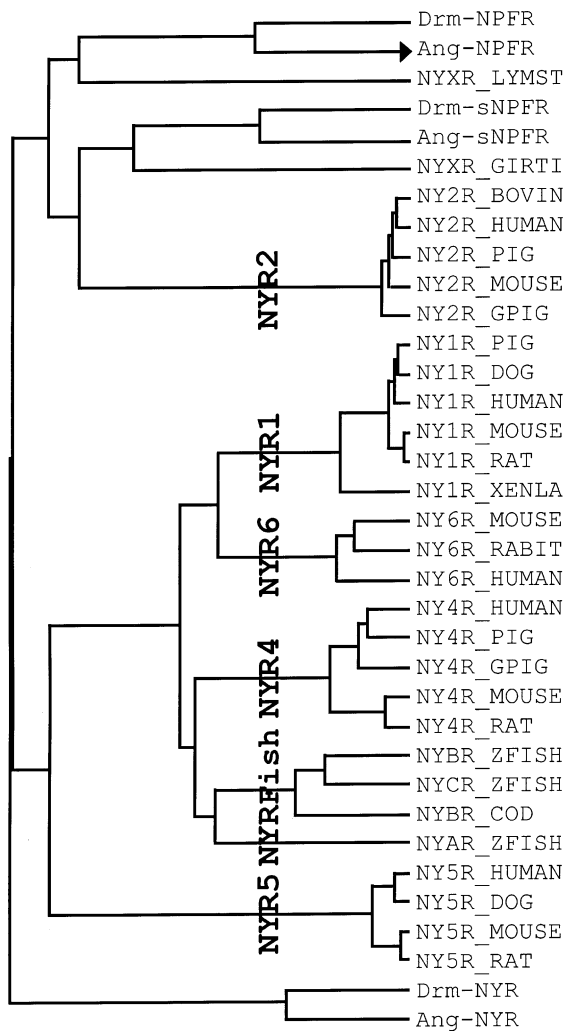


Fig. 6. Phylogenetic tree of NPY receptors. Sequences were subjected to Clustal X alignment; the dendrogram was constructed based on Jukes–Cantor distance analysis of the aligned sequences within GCG. For Ang-NPFR, the amino acid sequence was deduced from the nucleic acid sequence determined in the present study (cf. Fig. 1); other receptor sequences were obtained from GenBank and the *A. gambiae* and *D. melanogaster* genome projects.

NPF recognition by dipteran receptors, as well as the activity of the N-terminally truncated Drm-NPF_{8–36}. Also included for testing was Hez-MP-I, a lepidopteran heptapeptide [15], which is almost identical to the carboxyl terminal of Ang-NPF and Aea-NPF.

For Ang-NPFR, activities of peptides in binding assays indicate that species relatedness determines activities of full-length NPFs and suggest that N-terminal truncations may be unfavorable (Table 1). Ang-NPF was the most potent competitor of ¹²⁵I-Ang-NPF binding to isolated membranes, with an IC₅₀ of ~3 nM. Aea-NPF was almost as active (IC₅₀ ~ 10 nM), but Drm-NPF was less so (IC₅₀ ~ 97 nM). Accordingly, activity of full-length NPFs closely tracked structural relatedness. Drm-NPF_{8–36} was substantially less potent as a competitor (IC₅₀ 202 nM), suggesting that some

structural feature in the N-terminal one-third of NPFs is important for receptor interactions. Hez-MP-I was inactive, even when tested at a concentration of 1 μM, suggesting that the carboxyl terminal heptapeptide of Ang-NPF likely is insufficient for receptor activation.

Similar conclusions were reached when NPFs and related peptides were tested on membranes from cells stably expressing Drm-NPFR. The cognate ligand, Drm-NPF, was the most potent (IC₅₀ ~ 2 nM), with an activity comparable to Ang-NPF and its receptor (see above). Drm-NPF_{8–36} (IC₅₀ ~ 11 nM) lagged in activity, again indicating a functional role for the N-terminal. The clear difference between Ang-NPF (IC₅₀ ~ 8 nM) and Aea-NPF (IC₅₀ 87 nM) indicates that some structural features shared between Ang-NPF and Drm-NPF may be important for the binding of the latter to its receptor. Hez-MP-I again lacked activity. For comparison, the estimated binding activity of Drm-NPF was higher when membranes were assayed than when binding was tested utilizing whole cells [12]. In transiently transfected, intact HEK cells, Drm-NPF has been shown to promote recruitment of GFP-labeled β-arrestin to Drm-NPFR, but not to any of ten other *D. melanogaster* GPCRs tested [16], further confirming the activity of this ligand-receptor.

Neither of the other two GPCRs identified from the *A. gambiae* genome as related to the NPY receptor family (Fig. 6) are likely to be activated by Ang-NPF. The most distantly related of these (termed Ang-NYR) is similar to a *D. melanogaster* receptor (Drm-NYR, CG5811) that has been shown preliminarily [31] to be activated by small peptides wholly unrelated to NPFs or other NPY family members. The other *A. gambiae* receptor is identified as Ang-sNPFR based on its clear structural resemblance to Drm-sNPFR (CG7395). Recently, short neuropeptide Fs (sNPF; [36]) from *D. melanogaster* have been demonstrated as the ligands for Drm-sNPFR [11,21], which is capable of activating G-protein-coupled inwardly rectifying potassium channels in a pertussis-toxin sensitive manner [23]. A gene encoding Ang-sNPFs identified in the *A. gambiae* genome [24] shows little structural relation to the *Ang-NPF* gene. Indeed, peptide sequence similarities between NPFs and sNPFs are minimal, yet the protein sequences of their receptors are closely related.

Intriguingly, Ang-sNPFs resemble “*Aedes* head peptides” (Aea-HPs; [19,32]), one of which has been shown to inhibit host-seeking by female yellow fever mosquitoes [4]. The gene for Aea-HPs encodes a prohormone containing three copies of a decapeptide processed into a bioactive form [32], whereas the prohormones in *sNPF* genes of *A. gambiae* and *D. melanogaster* encode five or four peptides, respectively, with differing—but related—sequences. In adult female *A. aegypti*, the *Aea-HP* gene is expressed predominantly in brain, terminal ganglion, and midgut, but after a blood meal, the transcript expression patterns differ for each tissue during a gonotrophic cycle [32]. To date, no ortholog of the *Aea-HP* gene has been identified in *A. gambiae* or *D. melanogaster*, and nothing is known about the expression

of the *sNPF* gene in *A. gambiae*. The genes encoding NPF, sNPFs, and Aea-HPs in these dipterans have different structures, thus indicating that they apparently did not arise from a single ancestor gene, in contrast to their GPCRs.

As shown in this study by RT-PCR, transcripts for Ang-NPF and Ang-NPFR were present in the last larval instar, pupae, and both adult sexes, but only Ang-NPF transcripts were detected in embryos (eggs, Fig. 2). For both transcripts, the PCR products appear much diminished in the thorax of male and female *A. gambiae*, in comparison to that of larvae and pupae, and a reduction also is evident for male abdomen relative to that of female. The presence of Ang-NPF transcripts in head, thorax, and abdomens of the different stages presumably reflects gene expression in the brain (head) and ventral nerve cord (thorax and abdomen), but their presence in abdomen also may indicate gene expression in midgut. In both *D. melanogaster* and *A. aegypti*, NPF gene expression occurs in specific cells within the brain/CNS and midgut endocrine system [3,30]. Although no function for Aea-NPF has yet been determined, it clearly has a hormonal role in female *A. aegypti*, since it was isolated from hemolymph of blood-fed females, and its hemolymph titer changed during a gonotrophic cycle [30]. In general, expression of the Ang-NPFR gene shows the same pattern in life stages as that of the Ang-NPF gene (Fig. 2), and the presence of transcripts in the different body regions indicates gene expression likely occurs in the CNS, as shown for Drm-NPFR [39]. Detailed localization of Ang-NPFR gene expression in other tissues, along with fragments of information from other insects and invertebrates, likely will suggest possible links between NPF-related receptors and feeding, digestion, and reproduction in mosquitoes.

Insects exert many of their harmful actions through feeding-related activities, either as crop pests or as disease vectors. Attempts to implicate NPY family members in such activities date back to the early demonstrations of cells immunoreactive for PP in the CNS (e.g., blowfly; [9]) and midgut (cockroach; [10]) and have included studies on several non-dipteran species (e.g., [5,26,27,29,37]). NPY receptors have even been implicated in regulation of feeding in *Caenorhabditis elegans* [7], although the ligands (e.g., GLGPRPLRFa, termed AF9) recently described [17,25] for this nematode receptor initially do not appear to be members of either the NPF or sNPF peptide families. The emerging paradigm in mammals is relatively complex, with NPY exerting orexigenic responses centrally, whereas PYY₃₋₃₆ secreted from the gut is anorexigenic [22]. Promisingly, Drm-NPF and Drm-NPFR have been strongly tied to coordination of physiological processes dependent on nutritional status in *D. melanogaster* [28,39]. In mosquitoes such as *A. gambiae*, the blood meal plays a decisive role in determining the initiation of vitellogenesis, completion of oogenesis, and consequent reproductive success. Neuropeptides of the NPY family and their receptors likely participate in signaling in relation to nutritional status in the African malaria mosquito.

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